Circulating Antibody-Secreting Cell Response During Mycoplasma pneumoniae Childhood Pneumonia

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Abstract: BACKGROUND We recently demonstrated that the measurement of Mycoplasma pneumoniae (Mp)-specific immunoglobulin (Ig)M antibody-secreting cells (ASCs) improved diagnosis of Mp infection. Here, we aimed to describe Mp ASC kinetics and duration in comparison to conventional measures such as pharyngeal Mp deoxyribonucleic acid (DNA) and serum antibodies. METHODS This is a prospective longitudinal study of 63 community-acquired pneumonia (CAP) patients and 21 healthy controls (HCs), 3-18 years of age, from 2016 to 2017. Mycoplasma pneumoniae ASCs measured by enzyme-linked immunospot assay were assessed alongside Mp DNA and antibodies during 6-month follow-up. RESULTS Mycoplasma pneumoniae ASCs of the isotype IgM were found in 29 (46%), IgG were found in 27 (43%), and IgA were found in 27 (43%) CAP patients. Mycoplasma pneumoniae ASCs were detected from 2 days to a maximum of 6 weeks after symptom onset, whereas Mp DNA and antibodies persisted until 4 months (P = .03) and 6 months (P < .01). Mycoplasma pneumoniae ASCs were undetectable in HCs, in contrast to detection of Mp DNA in 10 (48%) or antibodies in 6 (29%) controls for a prolonged time. The Mp ASC response correlated with clinical disease, but it did not differ between patients treated with or without antibiotics against Mp. CONCLUSIONS Mycoplasma pneumoniae-specific ASCs are short-lived and associated with clinical disease, making it an optimal resource for determining Mp pneumonia etiology.

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Circulating antibody-secreting cell response during *Mycoplasma pneumoniae* childhood pneumonia

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40-word-or-less summary of article's main point

Mycoplasma pneumoniae (*Mp*)-specific antibody-secreting cells (ASCs) are detectable in peripheral blood of children with *Mp* community-acquired pneumonia within the first 1–2 weeks upon disease onset, and its presence is short-lived and associated with clinical disease.

Abstract

Background: We recently demonstrated that the measurement of *Mycoplasma pneumoniae* (*Mp*)-specific IgM antibody-secreting cells (ASCs) improved diagnosis of *Mp* infection. Here, we aimed to describe *Mp* ASC kinetics and duration in comparison to conventional measures such as pharyngeal *Mp* DNA and serum antibodies.

Methods: Prospective longitudinal study of 63 community-acquired pneumonia (CAP) patients and 21 healthy controls (HCs), 3–18 years of age, from 2016–2017. *Mp* ASCs measured by enzyme-linked immunospot (ELISpot) were assessed alongside *Mp* DNA and antibodies during 6-month follow-up.

Results: *Mp* ASCs of the isotype IgM were found in 29 (46%), IgG in 27 (43%), and IgA in 27 (43%) CAP patients. *Mp* ASCs were detected from 2 days to maximum 6 weeks after symptom onset, while *Mp* DNA and antibodies persisted until 4 months (p=0.03) and 6 months (p<0.01). *Mp* ASCs were undetectable in HCs, in contrast to detection of *Mp* DNA in 10 (48%) or antibodies in 6 (29%) controls for prolonged time. The *Mp* ASC response correlated with clinical disease, but did not differ between patients treated with or without antibiotics against *Mp*.

Conclusions: *Mp*-specific ASCs are short-lived and associated with clinical disease, making it an optimal resource for determining *Mp* pneumonia etiology.

Keywords

Antibiotic; B cell; carriage; diagnosis; host immune response; kinetic; macrolide; serology; vaccination

Introduction

Mycoplasma pneumoniae (*Mp*) is a common bacterial pathogen of community-acquired pneumonia (CAP) in children [1]. The "gold standard" for diagnosing *Mp* infection is a ≥4-fold increase in antibody levels [2] but has low sensitivity [3] and is not helpful in acute clinical management as it requires acute and convalescent sera [4]. Single immunoglobulin (Ig) M levels against *Mp*, and *Mp*-specific polymerase chain reaction (PCR) on upper respiratory tract (URT) samples are currently used to diagnose *Mp* CAP [5, 6]. However, these diagnostic tests are also positive in asymptomatic carriers, and therefore are unable to differentiate between *Mp* infection and carriage [7]. We recently demonstrated that the measurement of *Mp*-specific IgM antibody-secreting cells (ASCs) by enzyme-linked immunospot (ELISpot) assay improved diagnosis of *Mp* infection: *Mp*-specific IgM ASCs were detectable in children with *Mp* CAP, but not in *Mp* carriers suffering from CAP caused by other pathogens or asymptomatic *Mp* carriers [8].

Previous work in vaccine studies has established that circulating antigen-specific B cell responses are more rapid and shorter lived than antibody responses [9, 10]. Antigen-specific B cells proliferate and differentiate after antigen exposure into ASCs and memory B cells [11, 12]. ASCs then circulate in the peripheral blood for up to two weeks before migrating to secondary lymphoid organs or bone marrow, or undergoing apoptosis [12, 13]. However, data on the human B-cell response during infection rather than following controlled antigen challenge via vaccination are scarce. In a longitudinal follow-up of our recent study [8], we aimed to describe the onset, kinetics, duration, and isotype of the antigen-specific plasmablast response following *Mp* infection in children in comparison to more conventional measures such as *Mp* DNA in the URT and serum antibody responses. We further evaluated the effect of different treatment regimens against *Mp* on the ASC response and these other measures.

Methods

Ethics statement

The ethics committee of Zurich, Switzerland, approved the protocol for this study (no. 2016-00148). Written informed consent was obtained from all parents and from children from 14 years of age.

Participants

We consecutively enrolled patients between May 1, 2016, and April 30, 2017, at University Children's Hospital Zurich. In all children pharyngeal swab specimens were taken. Blood samples were collected if additional consent was given. All participants were invited to participate in this longitudinal follow-up study with additional visits between enrollment–2 weeks, 2 weeks–2 months, and/or 2–6 months after presentation.

CAP patients

CAP was defined as a clinical diagnosis of pneumonia in previously healthy children aged 3–18 years, as detailed elsewhere [8, 14]. Trained physicians identified cases at the emergency department and on pediatric wards. CAP patients <3 years of age were excluded to reduce the probability of viral infection, as it is highest in this age group [1, 15-17]. CAP patients were managed by treating physicians, who were not aware of the study test results, and treated according to current guidelines [5, 6].

Healthy controls (HCs)

HCs included healthy children undergoing elective surgical procedures and siblings of CAP patients. Healthy children undergoing elective surgical procedures were age-matched and excluded if there was a history of a recent (≤1 week) respiratory tract infection. Samples were collected at enrollment by the attending anesthesiologist before surgery while the child was

under general anesthesia. We also recruited and followed siblings of CAP patients without evidence of recent respiratory tract infection.

Study procedures

Collection of biological samples

Swabs were taken from the posterior pharynx using flocked nylon fiber tip swabs (Copan Diagnostics, Murrieta, CA, USA). Blood samples were collected in anticoagulated lithium heparin blood collection tubes and fresh peripheral blood mononuclear cells (PBMCs) were isolated ≤4 h after sampling to avoid poor assay performance due to decreased ASC viability. PBMCs were isolated by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and viability assessed by trypan blue exclusion. Serum was stored at –80°C.

Mp real-time PCR

DNA isolation was performed on pharyngeal swab samples with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A quantitative TaqMan (Applied Biosystems, Foster City, CA, USA) real-time PCR assay was used to detect and quantify *Mp* DNA as described previously [18].

Mp-specific enzyme-linked immunosorbent assay (ELISA)

Mp-specific IgM, IgG, and IgA antibody levels were determined using a commercially available ELISA (Virion\Serion, Würzburg, Germany), according to the manufacturer's instructions.

Mp-specific ASC ELISpot assay

The frequency of circulating *Mp*-specific IgM ASCs was measured by ELISpot assays using fresh PBMCs, as described previously [19], with some modifications. Briefly, 96-well ELISpot filter plates (Millipore, Billerica, MA, USA) were coated for 90 min at 37°C with the different antigens diluted in sterile phosphate-buffered saline (PBS). The antigens were: detergent extract of *Mp* enriched for highly specific adhesion protein P1 (2 µg/ml; Virion\Serion, Würzburg, Germany); Fluarix® Tetra quadrivalent influenza A and B virus vaccine (6 µg/ml; GlaxoSmithKline, Middlesex, UK); and affinity-purified antibodies to human Ig light chains (λ and κ, 10 µg/ml; Southern Biotech, Birmingham, AL, USA) as the positive control. The negative control consisted of PBS only in uncoated wells. After washing, coated plates were blocked with medium for another 90 min at 37°C. Coated plates were incubated at 37°C for 16–20 h with 100,000 or 10,000 viable PBMCs, and each dilution was used in triplicate. Plates were then washed, incubated with biotinylated anti-IgM, -IgG, -IgA and alkaline phosphatase (AP)-conjugated streptavidin (all Southern Biotech), and spots visualized using an AP substrate kit (Bio-Rad Laboratories, Hercules, CA, USA), with each spot appearing at the former location of a single ASC. Spots were counted by an ELISpot reader (AID, Strassberg, Germany) using pre-defined settings. The spots identified by the machine were manually inspected for the presence of artifacts. Antigen-specific spot counts were calculated as the mean of three wells minus the mean number of spots in PBS wells. Data were expressed as ASCs per 10^6 viable PBMCs [19].

Statistical analysis

We report dichotomous variables as percentages and continuous variables as medians with interquartile ranges (IQRs). The Mann-Whitney *U* test was used to compare medians, and the Fisher's exact test to compare proportions. Spearman rank correlation was used to evaluate relationships between variables. Kinetics were plotted with smooth curves, fitted by loess

(with a span of 0.67) using the scatter.smooth formula [20]. Study design and sample size considerations were described in detail previously [8]. All reported *p*-values are 2-tailed with statistical significance defined as *p*-value <0.05. Data were analyzed using the R software environment (version 3.6.0) [20].

Results

Study population

During the 12-month study period, we screened 450 CAP patients and 156 HCs **(Figure 1)**. A total of 152 CAP patients and 156 HCs were enrolled **(Table 1)**. *Mp*-specific PCR and serological test results of the enrolled population are shown in **Table 2**. Thereof, we included 63 CAP patients and 21 HCs with fresh (isolated ≤4 h) PBMCs available for additional testing with the *Mp*-specific ASC ELISpot assay, as previously reported [8]. There were no statistically significant differences between included and overall enrolled CAP patients and HCs in regards to age, sex, and season at enrollment **(Table 1)**. However, the proportion of siblings of index patients among HCs was higher in the included group of controls compared to enrolled controls (43% vs. 12%, $p<0.01$). Clinical, radiological, and laboratory characteristics of included CAP patients have been described separately [14, 21].

Kinetics and duration of the ASC response

We first assessed the ASC response at presentation and found *Mp*-specific ASCs in CAP patients ($n=63$) of the isotype IgM, IgG, and IgA in 29 (46%), 27 (43%), and 27 (43%), respectively **(Table 2)**. These first samples were collected at a median of 12 days after onset of symptoms (IQR 11–16, range 2–29). Then, the median number of *Mp* IgM ASCs was 690 (IQR 200–1,933) spots per 10^6 PBMCs, which was higher than that of *Mp* IgG ASCs (median 300, IQR 166–533; $p=0.07$) and *Mp* IgA ASCs (median 167, IQR 67–333; $p<0.01$). *Mp* IgM ASCs correlated with *Mp* IgG ASCs (rho 0.63, $p<0.01$) and *Mp* IgA ASCs (rho 0.62, $p<0.01$),

but not with *Mp* antibody (any isotype) or *Mp* DNA levels **(Supplementary Table 1)**. Time since onset of symptoms positively correlated with *Mp* IgM and *Mp* IgG antibody levels (rho 0.56 and 0.53, $p<0.01$), but not with *Mp* ASCs (any isotype), in first measured sample **(Supplementary Table 2)**. Among HCs ($n=21$), no *Mp* ASCs were detected, but *Mp* IgM serology was positive in 6 (29%) individuals, of whom 1 (5%) showed seroconversion to *Mp* IgG with a >4-fold increase in *Mp* IgG **(Table 2)**. All 29 *Mp* IgM ASC-positive CAP patients were also *Mp* PCR-positive and *Mp* IgM-positive, and the 3 (5%) *Mp* PCR-positive patients, who were *Mp* IgM ASC- and *Mp* IgM-negative, were identified as *Mp* carriers suffering from CAP caused by other pathogens, as detailed elsewhere [8]. The specificity of the *Mp* IgM ASC ELISpot assay was further demonstrated by the absence of *Mp* IgM ASCs in influenza virus [14] and Epstein-Barr virus (EBV) [22] infected patients **(Supplementary Figure 1)**. The 2 patients who tested positive for influenza virus in pharyngeal swab samples [14] had influenza-specific but not *Mp*-specific ASCs detectable during CAP.

The longitudinal follow-up study included 52 (62%) children (41 CAP patients and 11 HCs) with >2 visits in 42 (81%) and >3 visits in 27 (52%) children, performed at <2 weeks, 2 weeks–2 months, and 2–6 months in 43 (83%), 38 (73%), and 38 (73%) individuals, respectively. *Mp* ASCs were detected only within 6 weeks after symptom onset **(Figure 2)**, in contrast to *Mp* DNA ($p=0.03$) and *Mp* IgM and *Mp* IgG ($p<0.01$) persisting at 4 and 6 months, respectively **(Supplementary Table 3)**. The *Mp* IgA levels in CAP patients were low (median 11 U/ml, IQR 6–25) and above the cut-off in only 13 (21%) **(Table 2)**, and differed significantly from *Mp* IgM ($p<0.01$) and *Mp* IgG ($p<0.01$) levels. However, *Mp* antibody levels and kinetics correlated between isotypes **(Supplementary Table 1 and Figure 2)**. Detecting a ≥4-fold *Mp* IgG increase in *Mp* IgM ASC-positive patients was significantly more likely with *Mp* IgG <20 U/ml (91%, $n=10/11$) compared to *Mp* IgG ≥20 U/ml (0%, $n=0/18$; $p<0.01$) at presentation.

Interestingly, 3 siblings of CAP patients developed CAP during follow-up and were then also tested positive for *Mp* IgM ASCs. One of the 3 siblings was sampled (as asymptomatic sibling of a CAP patient) 3 days prior to the onset of symptoms, and at this time point, tested negative by the *Mp* ASC ELISpot assay, followed by a positive *Mp* ASC ELISpot assay result 6 days after developing symptoms and CAP diagnosis. Representative *Mp* IgM ASC ELISpot assay responses in this patient are shown in **Figure 3**. The kinetics of *Mp* DNA, *Mp* antibodies, and *Mp* ASCs of this patient are indicated in black in **Supplementary Figure 2**.

In HCs, *Mp* DNA and/or *Mp* IgM and IgG were detected for up to ≥2 months, while *Mp* ASCs and *Mp* IgA levels were negative during the complete 6-month follow-up period **(Figure 4)**. These results were in line with the lack of respiratory symptoms during follow-up visits.

Relation of the ASC response with clinical disease

We next compared the ASC response with clinical disease. As previously demonstrated in this cohort [14], *Mp* IgM ASC-positive CAP was statistically associated with prolonged prodromal symptoms and extrapulmonary manifestations, predominantly skin disorders [21]. However, the magnitude of the *Mp*-specific ASC response did not correlate with any of these clinical features **(Supplementary Table 2)**. Further, lower levels of C-reactive protein (CRP), white blood cell (WBC) count, absolute neutrophil count (ANC), and procalcitonin were statistically associated with IgM ASC-positive *Mp* infection [14]. *Mp* IgM ASC responses correlated positively with CRP levels (rho 0.45; p=0.01), and negatively with WBC count (rho -0.61; p<0.01) and ANC (rho -0.66; p<0.01), but not with other laboratory parameters **(Supplementary Table 2)**.

Next, we evaluated the effect of different treatment regimens against *Mp* on the ASC response. Ten (34.5%) patients received clarithromycin, 10 (34.5%) patients received doxycycline, and 9 (31%) patients were not treated with any antibiotic *in vitro* active against *Mp* (i.e., amoxicillin, *n*=5, amoxicillin-clavulanate, *n*=1, ceftriaxone, *n*=1, no antibiotic treatment, *n*=2). Kinetics of pharyngeal *Mp* DNA, *Mp*-specific antibodies, and *Mp* ASCs according to different treatment management are shown in **Figure 5**. There were no statistically significant differences between patients with or without treatment *in vitro* active against *Mp* with regards to ASC and antibody responses **(Supplementary Table 4)**. In contrast, *Mp* DNA was detected in patients treated with clarithromycin and doxycycline in 58% and 90% at 1–2-month follow-up (p=0.01) and 38% and 0% at 3–4-month follow-up (p=0.20), respectively. Notably, clinical outcome (length of hospital stay and long-term sequelae) and fever duration was not statistically different between treatment groups **(Supplementary Figure 3)**. Five patients who received antibiotics against *Mp* were additionally treated with corticosteroids because of severe extrapulmonary mucocutaneous manifestations [21]. *Mp* IgM ASC numbers did not differ between those patients before corticosteroid treatment and other CAP patients at an early time point (<2 weeks after onset of symptoms) (p=0.49), but were significantly lower at a later time point (2–4 weeks after onset of symptoms) following the administration of corticosteroids compared to CAP patients without corticosteroid treatment sampled at the same time period (p=0.04) **(Supplementary Figure 4)**.

Discussion

In this longitudinal follow-up study, we demonstrate that *Mp*-specific ASCs are short-lived and associated with clinical disease, in contrast to pharyngeal *Mp* DNA and serum antibodies that can persist for months and may be present also during *Mp* carriage. This study adds to our recent work [8] by detailing the onset, kinetics, duration, and isotype (IgM/IgG/IgA) of

the antigen-specific plasmablast response following *Mp* infection in children and investigating the effect of different treatment regimens against *Mp* on the specific immune response and pharyngeal *Mp* DNA load. These findings expand our current knowledge on specific B-cell responses to infection and provide an explanation for the high specificity and sensitivity of the *Mp* ASC ELISpot assay during *Mp* CAP [8]. The data presented give insight into disease pathophysiology and can therefore serve as a model for developing better diagnostic tests for other childhood infectious diseases.

We detected *Mp* ASCs as early as 2 days after the presentation of clinical symptoms, which was in line with the only previous study on *Mp*-specific ASCs including 12 *Mp*-seropositive children with CAP, in which *Mp* ASCs were detected within 5 days after symptom onset [23]. To our knowledge, we assessed also for the first time B-cell responses in a patient shortly prior to the development of symptoms and could thereby exemplarily demonstrate a specific ASC response upon CAP onset, which disappeared again with disease resolution, while *Mp* DNA and antibodies persisted for prolonged time. *Mp*-specific ASCs peaked around 1–2 weeks, similar to previous work: We recently reviewed the literature on the ASC response to infection [9] and found that the timing of ASC appearance in the blood is highly consistent after infection across several pathogens. ASCs are first detectable in peripheral blood at around 4 days following onset of symptoms before peaking at days 6–8 [9, 24]. In contrast to the conserved timing of ASC appearance, the magnitude of ASC responses varies widely between different bacterial and viral pathogens as well as between ASC isotypes [9]. Our results are consistent with previous work studying the immune responses to both vaccination [13] and infection [9], and demonstrate a synchronized response of B cells with different isotypes during infection. Conversely, serum antibody responses showed a sequential appearance in our study: IgM and IgA were first detectable followed by IgG with

concentrations that were still rising in the first 4 weeks following symptom onset, and lasting for several months.

However, in some individuals, B-cell responses could be detected up to 6 weeks following onset of symptoms indicating that *Mp*-specific ASCs may circulate longer compared with other (respiratory) infections, in which antigen-specific ASCs disappeared in most cases already 14 days after symptoms onset [9, 25-30]. These findings support the clinical observation of slow disease progression in *Mp* CAP [14], which may result in a longer antigen exposure and therefore immune activation allowing to detect *Mp*-specific ASCs in all patients during CAP. A more prolonged antigen exposure with slow disease progression may also be the reason for the significantly increased *Mp* IgG levels already in the first serum sample of *Mp* IgM ASC-positive CAP patients. The development of a further increase of *Mp* IgG in those patients ≥4-fold was therefore very unlikely which continues to question its value as "gold standard" for diagnosing *Mp* infection [3, 4]. Its interpretation is also complicated by our observation of a >4-fold increase in *Mp* IgG level in an asymptomatic sibling.

Besides describing *Mp* ASC response kinetics and duration, we also determined antibody specificity and isotype of the plasmablast response to infection, which are key to develop accurate ASC measurements [9]. We showed that *Mp*-specific ASCs were not detectable in patients infected with influenza virus or EBV, while patients with influenza virus infection on the other hand developed influenza virus-specific ASCs during CAP. The detection of *Mp* IgM ASCs was more sensitive than the detection of *Mp* IgG ASCs or *Mp* IgA ASCs for determining *Mp* infection, again in line with previous work [23].

312 The *Mp*-specific ASC response was associated with clinical disease and correlated with CRP
313 levels and WBC counts. Although *Mp* CAP was associated with lower CRP levels compared
314 to CAP of other origin [14], severe disease defined as the presence of extrapulmonary skin
315 manifestations was associated with increased systemic inflammation and higher CRP levels
316 [21]. This finding suggests that in these children, inflammation may be indeed driven by *Mp*
317 antigens, which warrants further investigation. Interestingly, there were no differences in ASC
318 or antibody responses, or clinical outcomes such as fever duration, between groups with
319 different antibiotic treatment regimens. In contrast, corticosteroid treatment significantly
320 decreased ASC responses, although they were still detectable. One-third of *Mp* IgM ASC-
321 positive CAP patients were not treated with an antibiotic *in vitro* active against *Mp*, but all of
322 them equally and fully recovered. Although the study design does not allow conclusions
323 about the effectiveness of treatment these findings highlight the need for future interventional
324 studies to assess the efficacy of antibiotics for *Mp* CAP [2, 31].
325
326 The ASC ELISpot assay is a robust technique [9, 32], and the protocol described here could
327 be translated directly into the clinical setting to diagnose *Mp* infection by using only a small
328 volume of peripheral blood (≥1 ml). Yet, the ASC ELISpot assay is labor-intensive requiring
329 the handling of fresh or frozen PBMCs [19, 32] and has a rather long overall turnaround time
330 (~24 h), but recent alternative protocols suggest more rapid (~6–8 h) ASC detection [19].
331 Optimizing such protocols will help to routinely use the *Mp* IgM ASC ELISpot assay for
332 diagnosing *Mp* CAP.
333
334 Despite variation in sampling time points, the study population represents a well-defined
335 cohort of children, both patients and controls, in whom pharyngeal swabs and fresh blood
336 samples at several time points up to 6 months following inclusion could be obtained. It is
337 challenging to collect blood samples from healthy children and already recovered children

and we can therefore not fully exclude any selection bias that occurred in the included subgroup of study participants. However, baseline characteristics of both included CAP patients and controls were similar compared with the enrolled study population. Some of the siblings of index patients were included as controls, which made it possible to increase the likelihood of detecting asymptomatic *Mp* carriers and to investigate the pattern symptom acquisition and/or changes in the results of the diagnostic tests. *Mp* was indeed more frequently detected in the URT of siblings compared to HCs sampled during elective surgery (32% vs. 4%), which is in line with a higher *Mp* transmission rate within families and between other close contacts [14, 33, 34]. Proportionally, more siblings than other controls were included than were initially enrolled into the study (43% vs. 12%), which may be partly due to a higher motivation of family members of affected patients agreeing to multiple sampling and follow-up. This may also explain the differences in *Mp* detection rates between included and enrolled study participants. A larger prospective confirmatory study is needed to validate these promising results of the *Mp* ASC ELISpot assay and to examine the added clinical utility of the ASC ELISpot assay to diagnose *Mp* infection in the clinical care of children with CAP.

In conclusion, *Mp*-specific ASCs are short-lived and associated with clinical disease, in contrast to pharyngeal *Mp* DNA and serum antibodies. There are limited data on the duration of B-cell responses during infection and our study indicates that *Mp*-specific peripheral blood B cells appear early in the course of illness, last only for several weeks before disappearing completely so that they can be easily detected in a clinical setting with good diagnostic sensitivity and specificity. These findings expand our current knowledge on specific B-cell responses to infection, and reveal ASCs as an optimal resource for determining disease etiology in *Mp* pneumonia and possibly other childhood infections.

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Conflict of interest

None.

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Author contributions

Study concept and design: P.M.M.S., A.M.C.v.R., C.B.; **Acquisition of data:** P.M.M.S.; **Analysis and interpretation of data:** P.M.M.S., J.T., A.M.C.v.R., C.B.; **Drafting of the manuscript:** P.M.M.S., J.T.; **Critical revision of the manuscript for important intellectual content:** all authors; **Statistical analysis:** P.M.M.S., J.T.; **Obtained funding:** P.M.M.S., A.M.C.v.R., C.B.; **Administrative, technical, or material support:** P.M.M.S., C.B.

References

1. Jain S, Williams DJ, Arnold SR, et al. Community-acquired pneumonia requiring hospitalization among U.S. children. N Engl J Med **2015**; 372:835-45.
2. Gardiner SJ, Gavranich JB, Chang AB. Antibiotics for community-acquired lower respiratory tract infections secondary to *Mycoplasma pneumoniae* in children. Cochrane Database Syst Rev **2015**; 1:CD004875.
3. Lee WJ, Huang EY, Tsai CM, et al. Role of serum *Mycoplasma pneumoniae* IgA, IgM, and IgG in the diagnosis of *Mycoplasma pneumoniae*-related pneumonia in school-age children and adolescents. Clin Vaccine Immunol **2017**; 24:e00471-16.
4. Waites KB, Xiao L, Liu Y, Balish MF, Atkinson TP. *Mycoplasma pneumoniae* from the respiratory tract and beyond. Clin Microbiol Rev **2017**; 30:747-809.
5. Harris M, Clark J, Coote N, et al. British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. Thorax **2011**; 66 Suppl 2:ii1-23.
6. Bradley JS, Byington CL, Shah SS, et al. The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America. Clin Infect Dis **2011**; 53:e25-76.
7. Spuesens EB, Fraaij PL, Visser EG, et al. Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. PLoS Med **2013**; 10:e1001444.
8. Meyer Sauteur PM, Seiler M, Truck J, et al. Diagnosis of *Mycoplasma pneumoniae* pneumonia with measurement of specific antibody-secreting cells. Am J Respir Crit Care Med **2019**; 200:1066-69.

9. Carter MJ, Mitchell RM, Meyer Sauteur PM, Kelly DF, Truck J. The antibody-secreting cell response to infection: kinetics and clinical applications. Front Immunol **2017**; 8:630.
10. Ellebedy AH, Jackson KJ, Kissick HT, et al. Defining antigen-specific plasmablast and memory B cell subsets in human blood after viral infection or vaccination. Nat Immunol **2016**; 17:1226-34.
11. Radbruch A, Muehlinghaus G, Luger EO, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol **2006**; 6:741-50.
12. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. Nat Rev Immunol **2015**; 15:160-71.
13. Blanchard-Rohner G, Pulickal AS, Jol-van der Zijde CM, Snape MD, Pollard AJ. Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans. Blood **2009**; 114:4998-5002.
14. Meyer Sauteur PM, Krautter S, Ambroggio L, et al. Improved diagnostics help to identify clinical features and biomarkers that predict *Mycoplasma pneumoniae* community-acquired pneumonia in children. Clin Infect Dis **2019**; Oct 26 [Epub ahead].
15. British Thoracic Society of Standards of Care Committee. British Thoracic Society guidelines for the management of community-acquired pneumonia in childhood. Thorax **2002**; 57:i1-24.
16. Cilla G, Onate E, Perez-Yarza EG, Montes M, Vicente D, Perez-Trallero E. Viruses in community-acquired pneumonia in children aged less than 3 years old: High rate of viral coinfection. J Med Virol **2008**; 80:1843-9.
17. Rhedin S, Lindstrand A, Hjelmgren A, et al. Respiratory viruses associated with community-acquired pneumonia in children: matched case-control study. Thorax **2015**; 70:847-53.

18. Hardegger D, Nadal D, Bossart W, Altwegg M, Dutly F. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. J Microbiol Methods **2000**; 41:45-51.

19. Saletti G, Cuburu N, Yang JS, Dey A, Czerkinsky C. Enzyme-linked immunospot assays for direct ex vivo measurement of vaccine-induced human humoral immune responses in blood. Nat Protoc **2013**; 8:1073-87.

20. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. **2019** Available at: http:// www.R-project.org/. Accessed 13 September 2019.

21. Meyer Sauteur PM, Theiler M, Büttcher M, Seiler M, Weibel L, Berger C. Frequency and clinical presentation of mucocutaneous disease due to *Mycoplasma pneumoniae* infection in children with community-acquired pneumonia. JAMA Dermatol **2019**; Dec 18 [Epub ahead].

22. Azzi T, Lunemann A, Murer A, et al. Role for early-differentiated natural killer cells in infectious mononucleosis. Blood **2014**; 124:2533-43.

23. Iseki M, Takahashi T, Kimura K, Yamashita R, Sasaki T. Number of specific antibody-secreting cells in the peripheral blood among children with mycoplasma pneumonia. Infect Immun **1996**; 64:2799-803.

24. Fink K. Origin and function of circulating plasmablasts during acute viral infections. Front Immunol **2012**; 3:78.

25. Palkola NV, Pakkanen SH, Kantele JM, Rossi N, Puohiniemi R, Kantele A. Pathogen-specific circulating plasmablasts in patients with pneumonia. PLoS One **2012**; 7:e34334.

26. Palkola NV, Blomgren K, Pakkanen SH, Puohiniemi R, Kantele JM, Kantele A. Immune defense in upper airways: a single-cell study of pathogen-specific

plasmablasts and their migratory potentials in acute sinusitis and tonsillitis. PLoS One **2016**; 11:e0154594.

27. Huang KY, Li CK, Clutterbuck E, et al. Virus-specific antibody secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model. J Infect Dis **2014**; 209:1354-61.

28. Habibi MS, Jozwik A, Makris S, et al. Impaired antibody-mediated protection and defective IgA B-cell memory in experimental infection of adults with respiratory syncytial virus. Am J Respir Crit Care Med **2015**; 191:1040-9.

29. Lee FE, Falsey AR, Halliley JL, Sanz I, Walsh EE. Circulating antibody-secreting cells during acute respiratory syncytial virus infection in adults. J Infect Dis **2010**; 202:1659-66.

30. Lee FE, Halliley JL, Walsh EE, et al. Circulating human antibody-secreting cells during vaccinations and respiratory viral infections are characterized by high specificity and lack of bystander effect. J Immunol **2011**; 186:5514-21.

31. Biondi E, McCulloh R, Alverson B, Klein A, Dixon A. Treatment of mycoplasma pneumonia: a systematic review. Pediatrics **2014**; 133:1081-90.

32. Truck J, Mitchell R, Thompson AJ, et al. Effect of cryopreservation of peripheral blood mononuclear cells (PBMCs) on the variability of an antigen-specific memory B cell ELISpot. Hum Vaccin Immunother **2014**; 10:2490-6.

33. Dorigo-Zetsma JW, Wilbrink B, van der Nat H, Bartelds AI, Heijnen ML, Dankert J. Results of molecular detection of *Mycoplasma pneumoniae* among patients with acute respiratory infection and in their household contacts reveals children as human reservoirs. J Infect Dis **2001**; 183:675-8.

34. Walter ND, Grant GB, Bandy U, et al. Community outbreak of *Mycoplasma pneumoniae* infection: school-based cluster of neurologic disease associated with household transmission of respiratory illness. J Infect Dis **2008**; 198:1365-74.

487

Tables

Table 1. Baseline characteristics of CAP patients and controls.

Characteristics	CAP		HC		OR (95% CI)	*P*
	Included (*n*=63)	Enrolled (*n*=152)	**Included (*n*=21)**	Enrolled (*n*=156)		
			n=12 (57) elective surgery *n*=9 (43) siblings	*n*=137 (88) elective surgery *n*=19 (12) siblings		
Age (years), median (IQR)	**6.0 (4.4–10.2)**	5.7 (4.3–8.9)	**6.1 (4.9–7.9)**	5.9 (4.3–8.1)	–	0.91
Sex (male), *n* (%)	**39 (62)**	84 (55)	**17 (81)**	102 (65)	0.4 (0.1–1.4)	0.18
Season at enrollment, *n* (%):						
- Spring (March–May)	**11 (17)**	21 (14)	**5 (24)**	43 (28)	0.7 (0.2–2.9)	0.53
- Summer (June–August)	**13 (21)**	30 (20)	**9 (43)**	22 (14)	0.4 (0.1–1.2)	0.08
- Autumn (September–November)	**17 (27)**	37 (24)	**4 (19)**	17 (11)	1.6 (0.4–7.3)	0.57
- Winter (December–February)	**22 (35)**	64 (42)	**3 (14)**	74 (47)	3.2 (0.8–18.7)	0.10
Day-care or pre-/school attendance, *n* (%)	**63 (100)**	NA	**20 (95)**	NA	NA	0.25
Immunizations[1], *n* (%)	**52/55 (95)**	NA	**14/14 (100)**	NA	NA	0.95
Underlying disease, *n* (%)	**10 (16)**	32 (21)	**12 (57)**	137 (88)	0.1 (0.0–0.5)	**<0.01**
- ENT, *n*	**0**	4	**12**	137[2]		
- Asthma or history of wheezing, *n*	**2**	8	**0**	0		
- Cardiovascular, *n*	**0**	2	**0**	0		
- Gastrointestinal, *n*	**2**	2	**0**	0		
- Neurological, *n*	**2**	5	**0**	0		
- Other, *n*	**4**	11	**0**	0		

Abbreviations: CAP, community-acquired pneumonia; CI, confidence interval; ENT, ear, nose, and throat; HC, healthy control; IQR, interquartile range; NA, not available; OR, odds ratio.

Differences between included CAP and included HC children (in bold) were determined by the Mann-Whitney *U* test (medians) and Fisher's exact test (proportions). *P* values <0.05 are indicated in bold.

[1] "Immunizations" refers to being immunized per the national immunization schedule in Switzerland.

[2] Children with elective surgery at the division of otolaryngology (*n*=137): hyperplasia of adenoids (*n*=62); eustachian catarrh (*n*=26); cysts, fistulae, and sinuses (*n*=10); protruding ears (*n*=8); and others (*n*=31).

501 **Table 2.** *Mp*-specific test results of enrolled and included CAP patients and controls.

Diagnostic test	CAP *n* (%)	HC *n* (%)	OR (95% CI)	*P*
Enrolled cohort:	**(*n*=152)**	**(*n*=156)**		
***Mp*-specific DNA**	44 (29)	12 (8) 6/137 (4) elective surgery 6/19 (32) siblings	4.9 (2.4–10.6)	**<0.01**
***Mp*-specific antibodies**				
IgM +	39/97* (40)	15/139* (11)	5.5 (2.7–11.7)	**<0.01**
IgG +	39/97 (40)	16/139 (12)	5.1 (2.6–10.7)	**<0.01**
IgA +	24/97 (25)	0/139 (0)	NA	**<0.01**
IgM + IgG +	37/97 (38)	7/139 (5)	11.5 (4.7–32.3)	**<0.01**
IgM + IgA +	24/97 (25)	0/139 (0)	NA	**<0.01**
IgG + IgA +	23/97 (24)	0/139 (0)	NA	**<0.01**
IgM + IgG + IgA +	23/97 (24)	0/139 (0)	NA	**<0.01**
Included cohort:	**(*n*=63)**	**(*n*=21)**		
***Mp*-specific DNA**	32 (51)	10 (48) 4/12 (33) elective surgery 6/9 (66) siblings	1.1 (0.4–3.6)	1.00
***Mp*-specific antibodies**				
IgM +	32 (51)	6 (29)	2.6 (0.8–9.1)	0.08
IgG +	27 (43)	5 (24)	2.4 (0.7–9.4)	0.19
IgA +	13 (21)	0 (0)	NA	**0.03**
IgM + IgG +	26 (41)	5 (24)	2.2 (0.7–8.8)	0.20
IgM + IgA +	13 (21)	0 (0)	NA	**0.03**
IgG + IgA +	11 (17)	0 (0)	NA	0.06
IgM + IgG + IgA +	11 (17)	0 (0)	NA	0.06
Seroconversion:				
- IgM	0/38** (0)	0/11** (0)	–	1.00
- IgG	5/38 (13)	1/11 (9)	1.5 (0.1–78.7)	1.00
- IgA	5/38 (13)	0/11 (0)	NA	0.57
Class switch from IgM to IgG	7/38 (18)	1/11 (9)	2.2 (0.2–111.7)	0.66
Titer increase:				
- IgM ≥2-fold [3]	3/38 (8)	0/11 (0)	NA	1.00
- IgG ≥4-fold [2]	10/38 (26)	1/11 (9)	3.5 (0.4–170.1)	0.41
***Mp*-specific ASC**				
IgM ASC +	29 (46)	0 (0)	NA	**<0.01**
IgG ASC +	27 (43)	0 (0)	NA	**<0.01**
IgA ASC +	27 (43)	0 (0)	NA	**<0.01**
IgM ASC + IgG ASC +	27 (43)	0 (0)	NA	**<0.01**
IgM ASC + IgA ASC +	27 (43)	0 (0)	NA	**<0.01**
IgG ASC + IgA ASC +	26 (41)	0 (0)	NA	**<0.01**
IgM ASC + IgG ASC + IgA ASC +	26 (41)	0 (0)	NA	**<0.01**

502
503 **Abbreviations:** ASC, antibody-secreting cell; CAP, community-acquired pneumonia; CI, confidence interval; ELISpot, enzyme-linked immunospot; HC,
504 healthy control; Ig, immunoglobulin; NA, not available; OR, odds ratio.
505
506 Data are presented as no. (%). Differences between groups are indicated by the Fisher's exact test. *P* values <0.05 are indicated in bold. *Sera were available in
507 97 (64%) CAP patients and 139 (89%) controls. **Paired sera were available in 38 (60%) CAP patients and 11 (52%) controls.

Figures

Figure 1. Study profile. Recruitment and flow of (*A*) CAP patients and (*B*) healthy controls (HC). Abbreviations: CAP, community-acquired pneumonia; HC, healthy control; PBMC, peripheral blood mononuclear cell.

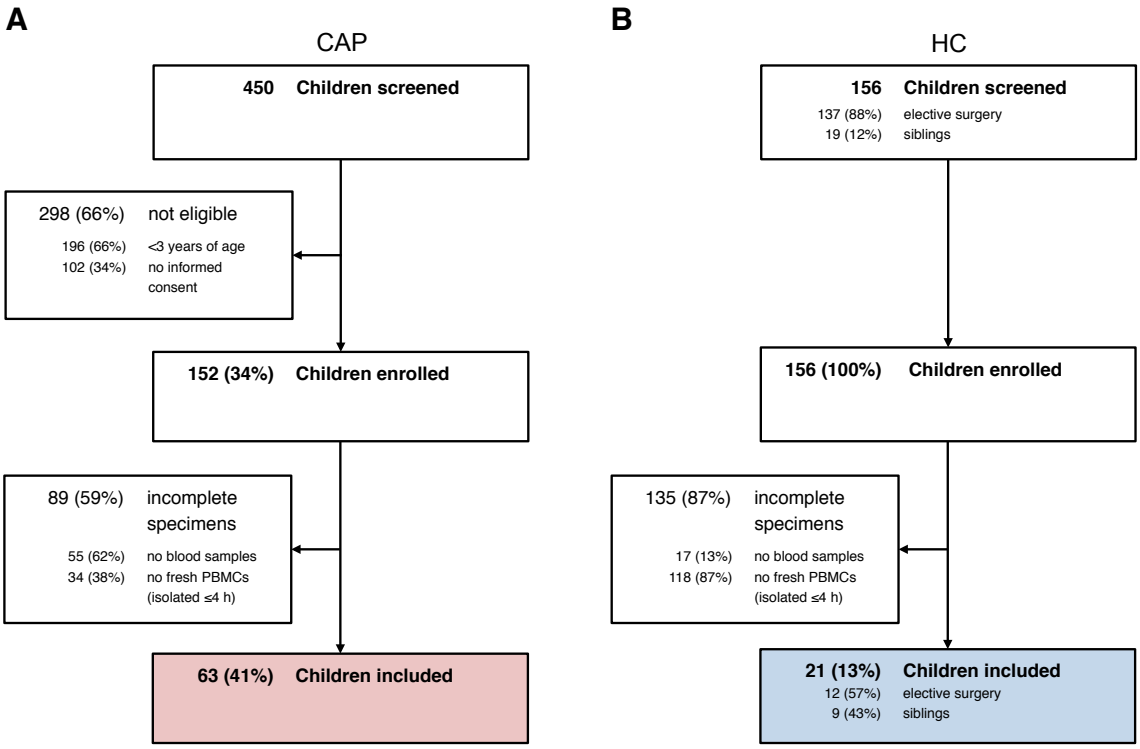

Figure 2. Kinetics of pharyngeal *Mp* DNA levels, *Mp* antibodies, and *Mp* ASCs in *Mp* IgM ASC-positive CAP patients. (*A*) *Mp* DNA levels in pharyngeal swab samples as genomic copy number per ml. (*B–D*) *Mp* antibodies and *Mp* ASCs of the isotype IgM (*B*), IgG (*C*), and IgA (*D*). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cut-off for the ELISA, lower limits of quantification for IgM, IgG, and IgA are 5, 3, and 2 U/ml. Abbreviations: ASC, antibody-secreting cell; Ig, immunoglobulin; *Mp*, *M. pneumoniae*; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction.

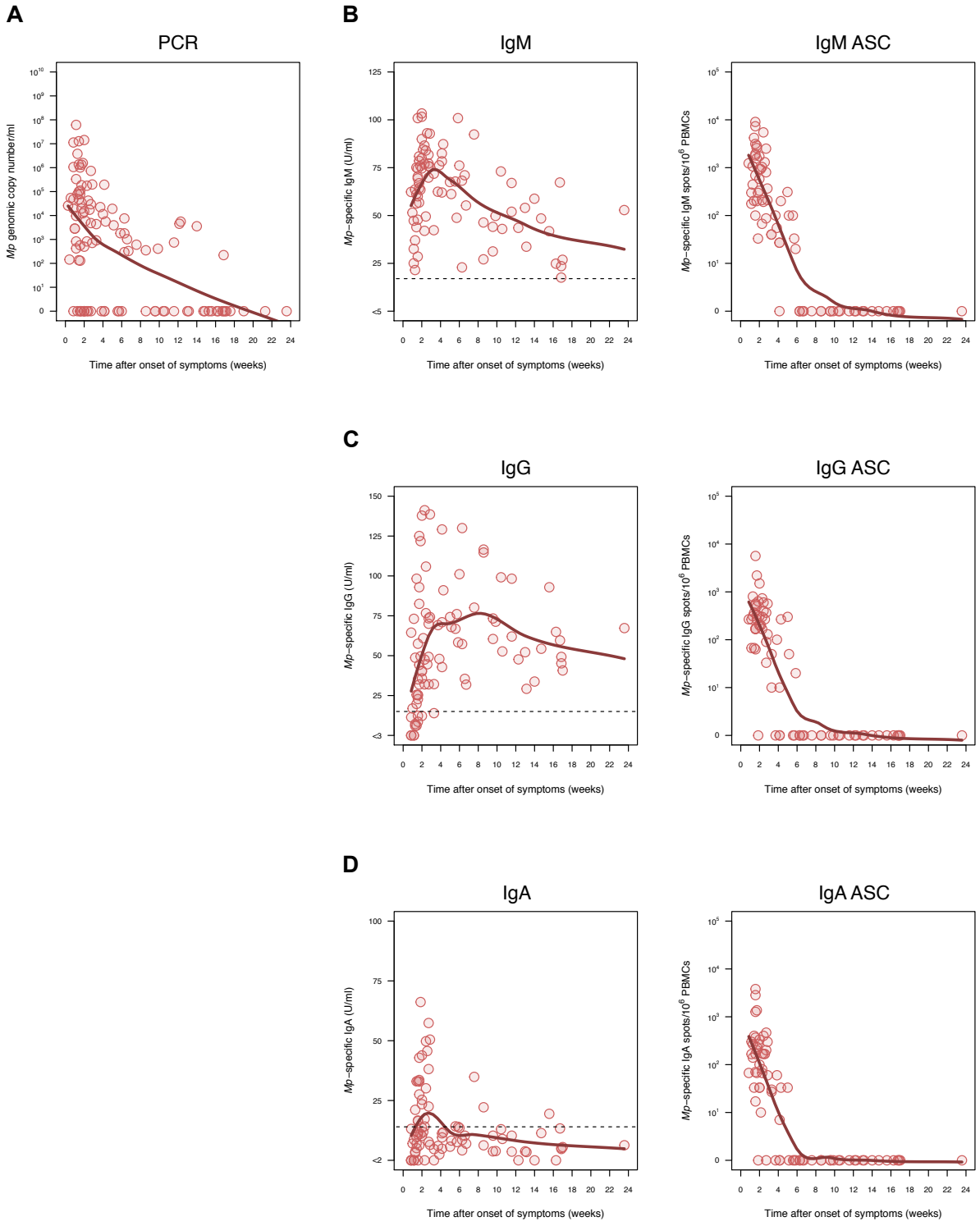
A
PCR
Mp genomic copy number/ml
Time after onset of symptoms (weeks)
B
IgM
Mp−specific IgM (U/ml)
Time after onset of symptoms (weeks)
IgM ASC
Mp−specific IgM spots/10^6 PBMCs
Time after onset of symptoms (weeks)
C
IgG
Mp−specific IgG (U/ml)
Time after onset of symptoms (weeks)
IgG ASC
Mp−specific IgG spots/10^6 PBMCs
Time after onset of symptoms (weeks)
D
IgA
Mp−specific IgA (U/ml)
Time after onset of symptoms (weeks)
IgA ASC
Mp−specific IgA spots/10^6 PBMCs
Time after onset of symptoms (weeks)

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Figure 3. Enzyme-linked immunospot (ELISpot) assay for ASCs in a patient who developed *Mp* CAP. Representative patterns of ELISpot assay wells 3 days prior to the onset of symptoms (*A*) and on day 6 (*B*) and day 81 (*C*) after symptom onset (100,000 PBMCs per well). Abbreviations: Ig, immunoglobulin; *Mp*, *M. pneumoniae*; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

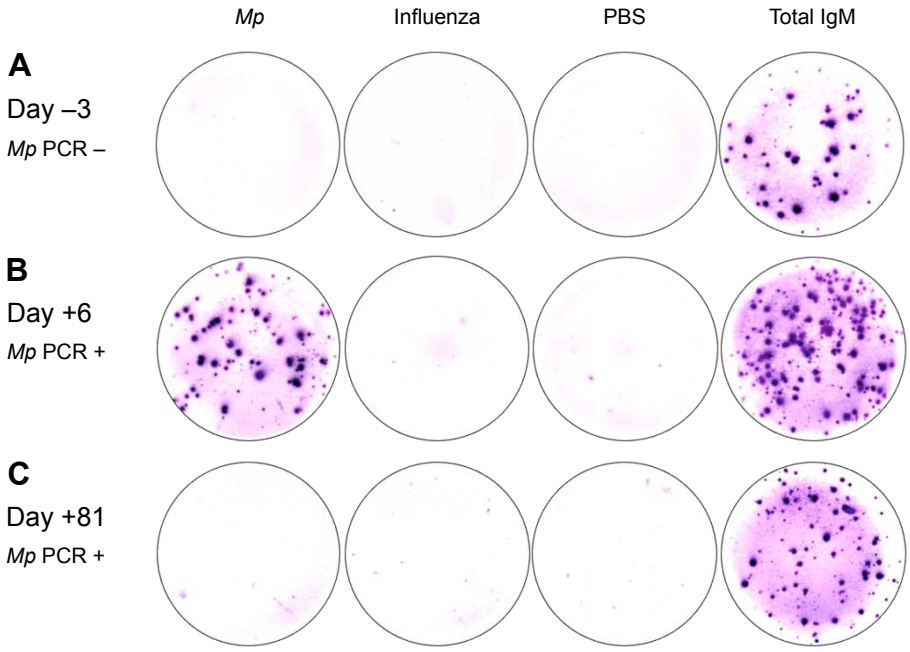


Figure 4. Kinetics of pharyngeal *Mp* DNA levels, *Mp* antibodies, and *Mp* ASCs in *Mp* PCR-positive controls. (*A*) *Mp* DNA levels in pharyngeal swab samples as genomic copy number per ml. (*B–D*) *Mp* antibodies and *Mp* ASCs of the isotype IgM (*B*), IgG (*C*), and IgA (*D*). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cut-off for the ELISA, lower limits of quantification for IgM, IgG, and IgA are 5, 3, and 2 U/ml. Abbreviations: ASC, antibody-secreting cell; Ig, immunoglobulin; *Mp*, *M. pneumoniae*; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction.

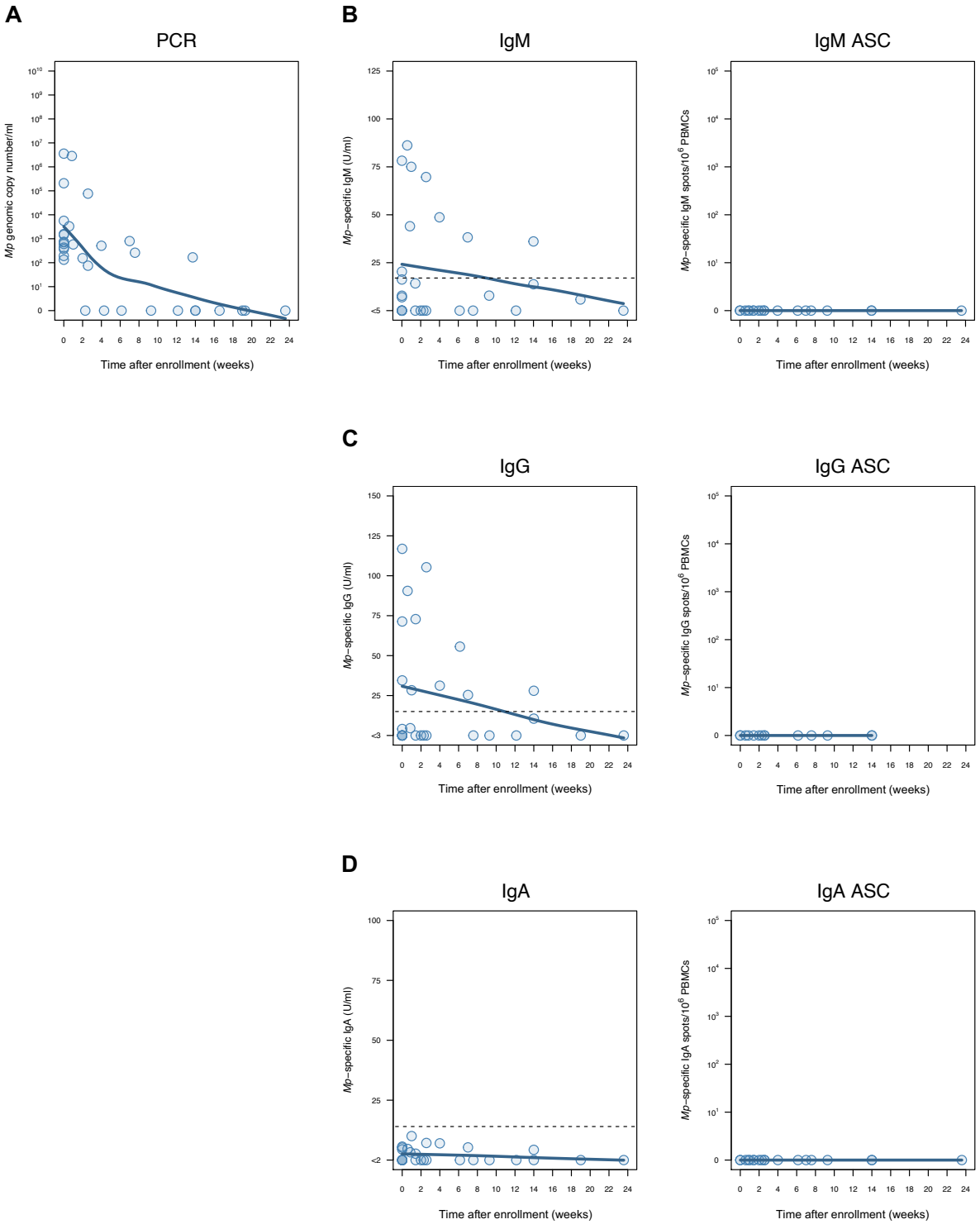
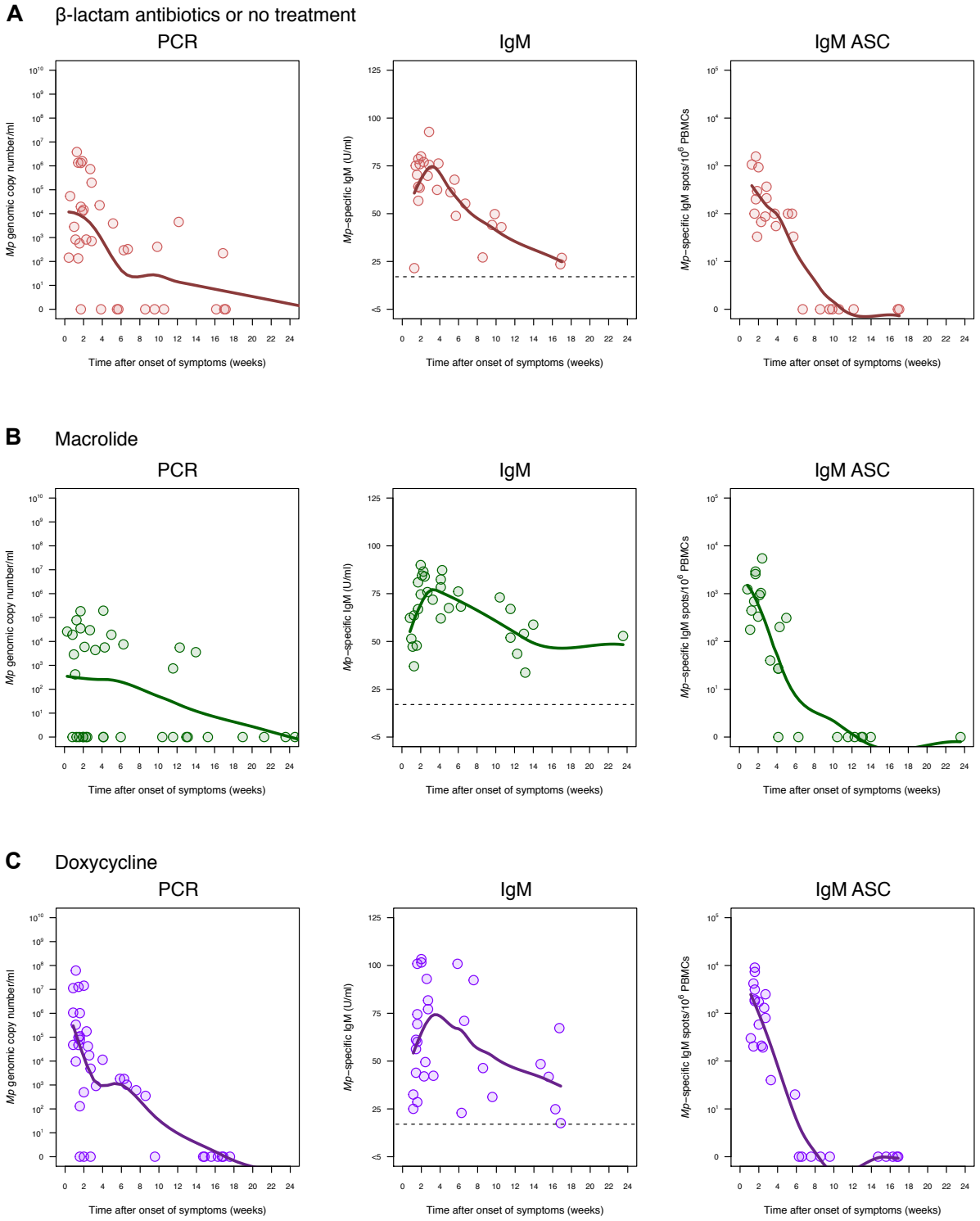

A
PCR
Mp genomic copy number/ml
Time after enrollment (weeks)
B
IgM
Mp−specific IgM (U/ml)
Time after enrollment (weeks)
IgM ASC
Mp−specific IgM spots/10^6 PBMCs
Time after enrollment (weeks)
C
IgG
Mp−specific IgG (U/ml)
Time after enrollment (weeks)
IgG ASC
Mp−specific IgG spots/10^6 PBMCs
Time after enrollment (weeks)
D
IgA
Mp−specific IgA (U/ml)
Time after enrollment (weeks)
IgA ASC
Mp−specific IgA spots/10^6 PBMCs
Time after enrollment (weeks)

Figure 5. Kinetics of pharyngeal *Mp* DNA levels, *Mp*-specific antibodies, and ASCs in *Mp* IgM ASC-positive CAP patients according to different treatment management. (*A*) β-lactam antibiotics (*n*=7) or no treatment (*n*=2). (*B*) Macrolides (Clarithromycin) (*n*=10). (*C*) Doxycycline (*n*=10). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cut-off for the ELISA (lower limits of quantification: 5 U/ml). Abbreviations: ASC, antibody-secreting cell; Ig, immunoglobulin; *Mp*, *M. pneumoniae*; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction.


A
β-lactam antibiotics or no treatment
PCR
IgM
IgM ASC
B
Macrolide
PCR
IgM
IgM ASC
C
Doxycycline
PCR
IgM
IgM ASC
Mp genomic copy number/ml
Mp–specific IgM (U/ml)
Mp–specific IgM spots/10^6 PBMCs
Time after onset of symptoms (weeks)

551